

10/517078

PCT J03/00706



REC'D 24 JUN 2003

WIPO PCT

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND
SALES hereby certify that annexed is a true copy of the Provisional specification
in connection with Application No. PS 2847 for a patent by UNIVERSITY OF
TECHNOLOGY, SYDNEY as filed on 07 June 2002.

WITNESS my hand this
Seventeenth day of June 2003

J Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES



BEST AVAILABLE COPY

AUSTRALIA

Patents Act 1990

University of Technology, Sydney

PROVISIONAL SPECIFICATION

Invention Title

*Novel screens to identify agents that modulate pericyte function
and diagnostic and therapeutic applications therefor*

The invention is described in the following statement:

Field of the invention

The present invention is in the field of drug screening, such as, for example agents that modulate cellular contractility or blood flow. More particularly, the present invention relates to novel screens for agents that modulate pericyte function, such as, for example, the contraction of pericytes, cell growth, differentiation, ion channel conductivity, neurotransmitter release, or gene transcription. The agents identified using such screens are useful for the diagnosis or therapeutic treatment of disorders involving impaired pericyte function, such as for example retinal oedema, glaucoma, retinopathy, or retinal neovascularisation in human or animal subjects.

Background of the invention

The retina of the eye comprises rod cells and cones as well as blood vessels (capillaries) and glial cells. Pericytes are localised on the abluminal wall of the capillaries in the retina, and extend processes down the long axis of, as well as around, the capillaries. Pericytes are contractile cells the contractions of which most likely regulate blood flow through the retinal microvasculature. This regulation of blood flow is mediated by the adhesion plaques, gap junctions and pericytic processes that communicate between the pericyte and the endothelial cells of the retina. Pericytic processes "attach" to several endothelial cells via so-called "peg and socket" junctions. Thus, the pericyte is in a unique position to influence flux across the microvasculature by affecting the "open" or "shut" state of endothelial intercellular junctions mechanically or by influencing endothelial cell contractile elements and/or endothelial cell transcytotic processes via humoral, ionic or other signal.

Direct evidence for a potential role in microvasculature tone control comes from the observations that pericytes in culture enter a contraction state as they grow (Kelly et al 1988, Jouce et al 1985). Functional evidence for the contractile ability of the pericytes was demonstrated using cells grown on a silicone rubber substrate, using a modification of the method of Harris et al (1980) Science Vol 208.

It is thought that microvascular pericytes are physiological regulators of fluid, nutrient, protein and hormone movement across the microcirculatory endothelial barrier and that the pathophysiology of several disease states including diabetes mellitus might be comprehended more fully when viewed in terms of alterations in a pericyte function. Diseases of the eye that can involve vascular complications include glaucoma, corneal angiogenesis, retinopathy of prematurity, and diabetic retinopathy. For example, impaired blood flow at and around the optic nerve head may be a major cause of complications associated with glaucoma.

10

In diabetic retinopathy, small blood vessels (capillaries) throughout the retina become damaged or blocked resulting in a lack of blood supply to small areas of the retina. Diabetes can thereby lead to changes in the permeability of the retinal vasculature. In more advanced cases of diabetic retinopathy, retinal neovascularisation can lead to leakage of blood into the retina, and retinal detachment, with consequent loss of vision. If diabetic eye disease is left untreated, it can lead to serious visual impairment or blindness.

Approximately 25% of diabetics have some degree of diabetic retinopathy. Diabetic retinopathy occurs in both Type I diabetics and Type II diabetics. Nearly all Type I diabetics will have evidence of diabetic retinopathy after twenty years and up to 21% of all Type II diabetics have retinopathy when they are first diagnosed with diabetes. Because Type II diabetes is often not diagnosed until the patient has had the disease for many years, diabetic retinopathy may be present in Type II patients at the time diabetes is discovered. In fact, many patients first learn that they have diabetes when their ophthalmologist finds diabetic retinopathy on a routine eye examination.

The diagnosis of retinopathy currently requires the invasive use of an ophthalmoscope and/or fluorescein angiography to assess the function of the capillaries of the retina. The procedure can also provide some confirmation of a preliminary diagnosis of diabetes. Unfortunately, there is currently no easily performed test for susceptibility of retinal capillaries to develop permeability defects.

35

Summary of invention

The present invention provides methods for determining or identifying compounds that modulate pericyte function, wherein a change in the contractile state of a pericyte is determined in the presence of a test compound, said
5 change indicating that the test compound modulates pericyte function. The compounds identified in the screens of the invention are those small molecules, peptides, proteins, hormones, nucleic acid, etc that agonise or antagonise pericyte contraction.

10 By "agonise pericyte contraction" is meant that the compound enhances the contraction of the pericyte, causing the pericyte to contract more fully or to a greater extent, or for a longer duration or more rapidly.

By "antagonise pericyte function" is meant that the compound reduces the
15 extent of contraction of the pericyte, thereby causing the pericyte to relax, or that the duration of any contraction of the pericyte is reduced or that the period of contraction is shortened or the relaxation phase of the cell is enhanced or prolonged.

20 By agonising or antagonising the contraction of the pericyte, the contractile state of the pericyte is modified. For example, a relaxed pericyte is caused to contract or a contracted pericyte is caused to relax. Similarly, a partially relaxed or partially contracted pericyte can be caused to relax more fully or for a prolonged period, or alternatively, to contract more fully or for a prolonged
25 period.

As used herein, the term "contractile state" shall be taken to mean the extent to which a pericyte is contracted. Accordingly, a reduced contractile state is one wherein the pericyte is more turgid or expanded or relaxed, or alternatively less
30 contracted or slower to contract or relaxed for a longer duration. Conversely, an enhanced or increased contractile state is one wherein the pericyte is more contracted or constricted or contracts more rapidly or remains contracts for a longer period.

35 The compounds identified in the novel screens of the invention are particularly useful in the diagnosis or therapeutic treatment of impaired pericyte function,

such as, for example, in cases of glaucoma, corneal angiogenesis, retinopathy of prematurity, or diabetic retinopathy. By way of exemplification, the inventors have identified several lead drugs that modify the contractile state of a pericyte. Preferably, such drugs are capable of enlarging the retinal capillaries and
5 thereby increase blood flow to the retina. Further, the inventors have developed a diagnostic test based on the potential for the small capillary blood vessels to have an impaired function in controlling blood flow in the eye. The effects of the drugs can be observed using recognised means according to a skilled person in the art. In a particularly preferred embodiment the diagnostic
10 test enables a practitioner to assess the risk of a subject developing glaucoma, corneal angiogenesis, retinopathy of prematurity, or diabetic retinopathy.

In particular, agonists of pericyte function are useful for the therapy of a condition wherein pericytes exhibit a reduced or impaired contractile force or
15 strength, or contract more slowly or for a shorter duration than the pericytes of a healthy subject. The application of such agonist compounds may be indicated in cases where there is an oversupply of blood to the retina, such as, for example, in cases of diabetic retinopathy.

20 Similarly, antagonists of pericyte function identified according to the inventive method are useful for the treatment of a condition wherein the pericytes are excessively stimulated to contract, or remain in a contracted state, or where there is a reduced supply of blood to the retina. Surgical intervention may also involve the use of such compounds.

25 Accordingly, a second aspect of the invention provides for the use of a compound that modulates pericyte function in the preparation of a medicament for the treatment of impaired pericyte function in a subject, said compound being identified by a process comprising determining a change in the
30 contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function.

In a related embodiment, there is provided a method for treating a subject having impaired pericyte function comprising administering to the subject an
35 amount of a pharmaceutical composition comprising a compound that modulates pericyte function and a pharmaceutically acceptable carrier, diluent

or excipient, wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function.

5

The compounds identified in the novel screens of the invention, especially those agonists of pericyte function, are particularly useful in the diagnosis of impaired pericyte function in a subject. Compounds that have been shown by *In vitro* screening to modulate pericyte function can be tested for safety and efficacy in animal models, such as for example, dogs or rats or pigs, and then proceed to clinical trials in humans, if desired. Naturally, for veterinary applications, no clinical trial in humans is required. Those compounds that are safe and efficacious in animals or humans can be applied to the eye of an appropriate subject to test for normal or healthy retina function. For subjects suffering from impaired pericyte function, such as, for example, glaucoma, corneal angiogenesis, retinopathy of prematurity, or diabetic retinopathy, agonist compounds will generally either fail to elicit a contraction when applied to the surface of the eye, or alternatively, elicit a slowed or incomplete contraction compared to the contractile state achieved for a healthy subject under similar or identical conditions.

Accordingly, a third aspect of the invention provides a method of diagnosing impaired pericyte function in a subject comprising administering to the subject an amount of a compound that modulates pericyte function under conditions sufficient to modify the contractile state of a pericyte and determining the change in the contractile state of the subject's pericytes, wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function. Preferably, the change in the contractile state of the subject's pericytes is determined by a comparison with the change in contractile state of a pericyte of a healthy subject.

In a related embodiment, the present invention provides a method of diagnosing retinal capillary damage in a subject, the method comprising:

- (i) administering to the subject an amount of a compound that modulates the contractile state of a pericyte wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function; and
- (ii) detecting dilation or constriction of a capillary in the retina of the subject, wherein a slow or unsubstantial dilation or constriction of the capillary indicates retinal capillary damage.
- 10 Preferably, the change in the contractile state of the subject's capillaries is determined by a comparison with the change in contractile state of a retinal capillary of a healthy subject.

Brief description of the drawings

- 15 Figure 1 shows a phase contrast image of pericytes (after 24hr growth) contacting a silicone substrate, obtained using a videocamera (SONY SSC-DC30P) and framegrabber (DT3100) attached to an inverted microscope (NIKON TE200). Contraction of the pericytes was observed as wrinkles induced in the silicone substrate. Wrinkles are indicated by the arrows. A
- 20 wrinkle was easily distinguished from the cell membrane by (i) a variable thickness along its length and (ii) marked changes in phase along its length. All of the pericytes in this field exhibit wrinkling of the silicone substrate.

- Figure 2 shows a phase contrast image of pericytes (after 24hr growth) contacting a silicone substrate, obtained using a videocamera (SONY SSC-DC30P) and framegrabber (DT3100) attached to an inverted microscope (NIKON TE200). Contraction of the pericytes was observed as wrinkles induced in the silicone substrate. Wrinkles are indicated by the arrows. A
- 30 wrinkle was easily distinguished from the cell membrane by (i) a variable thickness along its length and (ii) marked changes in phase along its length. All of the pericytes in this field exhibit wrinkling of the silicone substrate.

- Figure 3 is a graphical representation showing the dose-dependent effect of norepinephrine (NE) on the contraction of retinal pericytes. The concentration of NE is indicated on the x-axis. Contractility index, as determined from the
- 35 number of silicone wrinkles for pericyte cultures, is indicated on the abscissa.

NE intensified the number of silicone wrinkles in a contraction-dependent manner, inducing development of the contractile tone of the pericytes. NE at concentrations greater than 10^{-6} M caused contraction, with maximum enhancement of contractile state of the pericytes at 10^{-4} M NE. NE induced
5 pericyte contraction of silicone substrate in a dose-dependent manner ($EC_{50}=8\mu M$).

Figure 4 is a graph of the effect of Pituitary Adenylate Cyclase Activating Peptide (PACAP) on the contractility of pericytes. The EC_{50} concentration is
10 3nM, for these data averaged from 6 cells.

Figure 5 is a graph of the effect of Vasoactive Intestinal Peptide (VIP) on the contractility of pericytes. The EC_{50} concentration is 48nM, for these data averaged from 3 cells.

15 Figure 6 is a graph of the effect of different concentrations of Rp-cAMPS on the relaxation of pericytes that had been stimulated by PACAP (10^{-8} M). The numbers of pericytes included in the sample-size for each concentration is included in each bar.

20 Figure 7 is a graph of the effect of inhibiting phospholipase C on the PACAP-induced relaxation of pericytes. The conditions indicated by the bars are as follows: U=U73122 alone, UP=U73122 in the presence of PACAP, UPR=U73122 in the presence of both PACAP and Rp-cAMPS.

25 Figure 8 is a graph of the effect of different concentrations of N-phenylanthranilic acid on the relaxation of pericytes. The data are averaged from a sample of 7 pericytes. A contractility index of less than 100 indicates that the pericyte had relaxed in response to the drug.

30 Figure 9 is a graph of the effect of different concentrations of flufenamic acid on the relaxation of pericytes. The data are averaged from a sample of 4 pericytes. A contractility index of less than 100 indicates that the pericyte had relaxed in response to the drug.

35

Figure 10 is a graph of the effect of different concentrations of R-flurbiprofen on the relaxation of pericytes. The data are averaged from a sample of 8 pericytes. A contractility index of less than 100 indicates that the pericyte had relaxed in response to the drug.

5

Detailed description of the invention

One aspect of the present invention provides a method for determining or identifying a compound that modulates the contractile state of a pericyte comprising incubating a pericyte in the presence of a test compound and determining a change in the contractile state of a pericyte, wherein said change indicates that the compound modulates the contractile state of the pericyte.

Preferably, the contractile state of the pericyte is determined before the compound is incubated with the pericyte, thereby facilitating a determination of the change in contractile state of the pericyte. Alternatively, in certain preferred assay formats, the pericyte generally assumes a contracted state in the absence of a test compound, in which case relaxation of the pericyte or a reduced contractile state will be readily detectable.

20

Any art recognised means can be used to determine the contractile state of a pericyte, such as, for example, visual detection, second messenger assay involving a determination of cAMP levels or Ca^{2+} efflux, FACS analysis, ion channel activity, determining cellular permeability, or by determining a force that the cell applies to a surface with which the cell is in contact and the like.

The contractile state of a pericyte is preferably determined by visual means, such as, for example, employing a microscope or similar analytical tool wherein the size or volume of the cell *in vivo* or *in vitro* or *in situ*. Preferably, the inventive method employs a technique for visualization, maintenance or culture of pericytes such that their contraction or relaxation can be readily detected, and subjected to quantitation.

The inventive method clearly encompasses the sequential incubation of pericytes with a range of different compounds, that may be antagonistic towards each other. Cells may be washed between incubations to remove the

35

residual first compound or reduce the concentration of said compound to level that is too low to exert an effect on the contraction of the pericyte.

5 In an alternative embodiment, the method further comprises contacting the pericyte with a second test compound without washing the cells between incubations. In such cases, a second compound that is antagonistic towards the first compound will reverse the effects of the first compound if present at a sufficiently high concentration. Alternatively, where the second compound is an agonist of the first compound, the contractile state of the pericyte may be
10 enhanced step wise (ie enhanced contraction or enhanced relaxation) between incubations.

In a particularly preferred embodiment, the contractile state of pericytes is determined by their growth on a medium having a resilient or flexible support
15 that deforms when the contractile state of the pericytes in contact therewith is modified.

In a particularly preferred embodiment, the present invention provides a method of identifying a compound that modulates the contractile state of a
20 pericyte comprising

- (i) providing a pericyte cell in physical contact with a resilient support under conditions sufficient for a pericyte contraction to distort said level or planar resilient support;
- (ii) contacting a candidate compound with said pericyte cell; and
- 25 (iii) determining a distortion in said resilient support, wherein said distortion indicates that said compound modulates the contractile state of the pericyte.

As used herein "physical contact" means sufficiently adherent for a change in the contractile state of the pericyte to produce a distortion in the support.
30 Preferably, the cell is fixed to the support.

Preferably, the resilient support is a resilient sheet. The term "resilient" refers to the characteristic of being capable of withstanding permanent deformation or rupture, or being capable of recovering from or adjusting to change or
35 treatment. In the present context, the resilient support need only be sufficiently resilient to deform during contraction or relaxation of a pericyte that is adhered

thereto and to assume its normal appearance when the contractile state of the pericyte is modified. Highly elastic materials are not required for this purpose, since the extent of deformation of the support required is small.

- 5 Preferably the support is constructed of glass, or a polymeric material, such as, for example, a cross-linked polymer. Mixed supports of glass and polymeric materials are particularly preferred.

10 Preferably, the support comprises glass having a cross-linked polymer coating to which the pericytes are attached or fixed or otherwise in physical contact with. In one embodiment the support has a cross-linked silicone fluid layer.

15 Preferably the support is level or planar to facilitate the adherence or culture of a monolayer or bilayer of cells thereto. Use of an uneven support may, in certain circumstances hinder quantitation because of the greater difficulty of correlating a single distortion with a single cell's contractile state. By "level or planar" means sufficiently even to permit the growth or culture or adherence of an even layer of cells in connection therewith, such as, for example an approximate monolayer of cells or an approximate bilayer of cells.

20 Preferably the term "distort" as used herein means to change from the natural, normal, or original shape or condition.

25 When pericytes are cultured on very thin sheets of cross-linked polymer, the traction forces that the cells exert distort the polymer layer. Preferably, the distortion of the cell is visualised as elastic distortion or wrinkling of the polymer layer.

30 Preferably determining a distortion comprises measuring or otherwise determining the change in size or volume of the cell *in vivo* or *in vitro* or *in situ*. For example, the contractile index of a cell can be readily determined by counting the number of wrinkles or distortions of the support.

35 The inventive method clearly encompasses the sequential incubation of pericytes with a range of different compounds, that may be antagonistic towards each other. In one embodiment, the method further comprises:

- (i) washing the pericyte with a suitable buffer or aqueous solvent that is not damaging to the integrity or contractile function of the cell for a time and under conditions sufficient to remove the compound or reduce its activity to a level that does not affect pericyte contraction;
 - 5 (ii) contacting the pericyte with a second test compound ; and
 - (iii) determining a distortion in said resilient support, wherein said distortion indicates that said compound modulates the contractile state of the pericyte.
- 10 In an alternative embodiment, the method further comprises contacting the pericyte with a second test compound wherein a further distortion in the resilient support indicated that the compound modulates the contractile states of the pericyte. If this second distortion is opposed to the distortion obtained for the first test compound, then the second compound is an antagonist of the first
- 15 compound. If the second distortion amplifies the distortion achieved for the first test compound, then the second compound is an agonist of the first compound. For example, a first test compound may induce relaxation of the pericytes, thereby reducing wrinkling of the resilient support, whereas a second compound may induce pericyte contraction, thereby increasing the number of
- 20 wrinkles in the resilient support. On the other hand, if both the first and second compounds produce the same effect, the number of wrinkles in the resilient support may be enhanced in a step wise manner between the addition of the first and second compounds (for agonists of pericyte function) or reduced in a step wise manner (for antagonists of pericyte function). All such possibilities
- 25 are encompassed by the invention.

Preferably, the method further comprises comparing a visualised distortion of a cell from a subject with a control cell which can be treated (by contacting with a candidate compound) or untreated. In one embodiment, the control cell is a

30 healthy cell. In an alternate embodiment, the control cell is an unhealthy cell such as for example a cell obtained from a diseased patient or alternatively a cell that has been treated to produce a cell having impaired function.

In one embodiment, the method further comprises a competition type assay,

35 wherein, the method comprises contacting a competitive inhibitor compound with the pericyte cell either before or after the candidate compound is contacted

with the cell. Preferably, the competition compound modulates the contractile state to decrease or increase the contractile state of the pericyte.

Preferably, the inventive method further comprises formulating the compound
5 in a suitable pharmaceutically acceptable carrier or diluent or excipient, and more preferably administering the compound to a subject in need of treatment, such as, for example, a subject having impaired pericyte function or suspected of having impaired pericyte function.

10 A second aspect of the invention provides for the use of a compound that modulates pericyte function in the preparation of a medicament for the treatment of impaired pericyte function in a subject, said compound being identified by a process comprising determining a change in the contractile state
15 of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function.

Preferably the compound is selected from the group consisting of:
pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive
intestinal polypeptide (VIP), a compound having activity on phospholipase C
20 (PLC), a compound having activity on protein kinase A (PKA), a compound having activity on ion-channel hyperpolarisation channels, and a non-steroidal anti-inflammatory drug (NSAID). Preferably, the non-steroidal anti-inflammatory drug is N-phenylanthranilic acid or flufenamic acid or flurbiprofen.
More preferably, flurbiprofen will be the R-isomer form.

25 Homologues, analogues or derivatives of the compound identified using the screens referred to herein are clearly contemplated herein, the only requirement being that such homologues, analogues and derivatives retain the same activity with respect to pericyte function as the base compound from
30 which they are derived, and preferably retain the ability of the base compound to modulate pericyte contractile state.

In the case of proteinaceous compounds (ie peptides, polypeptides, enzymes and the like) functionally equivalent homologues are obtained from other
35 sources, such as, for example viruses, bacteria, related organisms. Synthetic peptides, are particularly contemplated herein.

- Particularly preferred modifications are those modifications designed to increase the stability of the identified peptides. Typical stabilising groups include amido, acetyl (eg at N-terminus), glycerol, benzoyl, phenyl, tosyl, alkoxy-carbonyl, alkyl-carbonyl, benzyloxycarbonyl and the like group modifications. Additional modifications include using an "L" amino acid in place of as "D" amino acid, cyclisation of the polypeptide, and amide rather than an amino or carboxy-terminal to inhibit exopeptidase activity.
- 10 Another approach to modification is to link the peptides or proteins to a variety of polymers, such as polyethylene glycol (PEG) and polypropylene glycol (PPG) - see for example US Patent Nos 5091176, US 5214131 and US 5264209.
- 15 The proteinaceous compounds, such as, for example, PACAP or VIP, can be synthesised by any of the techniques that are known to one of ordinary skill in the art, for example, synthetic chemistry techniques (eg solid phase synthesis for solution synthesis) and/or recombinant DNA techniques. Synthetic chemistry techniques (eg solid phase synthesis) may be preferred for reasons
- 20 of purity, freedom from an undesired side products and ease of product purification. Techniques for chemically synthesizing peptides of the invention are reviewed by Borgia and Fields, 2000, TibTech 18:243-256 and described in detail in the references contained therein.
- 25 Alternatively, the proteinaceous compounds, such as, for example, PACAP or VIP, can be produced by recombinant DNA techniques in a host cell transformed with the nucleic acid having a sequence encoding such peptide. To produce a peptide by recombinant techniques, host cells (eg bacterial cells such as *E.coli* insect cells, yeast, or mammalian cells, for example, Chinese
- 30 hamster ovary cells are transformed with a vector suitable for expressing a peptide of the invention and cultured in a medium such that the cells produce peptides). Peptides so-produced can be purified from cell culture medium, host cells, or both using techniques known in the art for purifying peptides including ultrafiltration, ion-exchange chromatography, gel filtration chromatography,
- 35 electrophoresis or immunopurification with antibodies specific for the peptide.

Preferably, proteinaceous compounds are purified substantially free of conspecific proteins.

- The compound is conveniently formulated in a pharmaceutically acceptable excipient or diluent, such as, for example, an aqueous solvent, non-aqueous solvent, non-toxic excipient, such as a salt, preservative, buffer and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Aqueous solvents include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art.
- Optionally, the compound formulation will also include a carrier, such as, for example, to reduce surface denaturation of the compound if it is present at a low concentration in the formulation. Commonly used carrier molecules are bovine serum albumin (BSA), ovalbumin, mouse serum albumin, rabbit serum albumin and the like. Synthetic carriers also are used and are readily available.
- Carriers may be conjugated to the active compound. Means for conjugating polypeptides to carrier proteins are also well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.
- It may also be desirable to co-administer biologic response modifiers (BRM) with the compound, to down regulate T cell responses or antibody responses to the compound.

- Preferred vehicles for administration of the compound include liposomes.
- Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. (Bakker-Woudenberg *et al.*, *Eur. J. Clin. Microbiol. Infect. Dis.* 12(Suppl. 1), S61 (1993); and Kim, *Drugs* 46, 618

(1993)). Liposomes are similar in composition to cellular membranes and as a result, liposomes generally are administered safely and are biodegradable.

Techniques for preparation of liposomes and the formulation (e.g.,
5 encapsulation) of various molecules, including peptides and oligonucleotides, with liposomes are well known to the skilled artisan.

Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and can vary in size with diameters ranging from 0.02 μm to
10 greater than 10 μm . A variety of agents are encapsulated in liposomes. Hydrophobic agents partition in the bilayers and hydrophilic agents partition within the inner aqueous space(s) (Machy *et al.*, LIPOSOMES IN CELL BIOLOGY AND PHARMACOLOGY (John Libbey 1987), and Ostro *et al.*, *American J. Hosp. Pharm.* 46, 1576 (1989)).

15

Liposomes can also adsorb to virtually any type of cell and then release the encapsulated agent. Alternatively, the liposome fuses with the target cell, whereby the contents of the liposome empty into the target cell. Alternatively, an absorbed liposome may be endocytosed by cells that are phagocytic.
20 Endocytosis is followed by intralysosomal degradation of liposomal lipids and release of the encapsulated agents (Scherphof *et al.*, *Ann. N.Y. Acad. Sci.* 446, 368 (1985)). Irrespective of the mechanism or delivery, however, the result is the intracellular disposition of the associated compound.

25 Liposomal vectors may be anionic or cationic. Anionic liposomal vectors include pH sensitive liposomes which disrupt or fuse with the endosomal membrane following endocytosis and endosome acidification. Cationic liposomes are preferred for mediating mammalian cell transfection *in vitro*, or general delivery of nucleic acids, but are used for delivery of other therapeutics, such as
30 compounds.

Cationic liposome preparations are made by conventional methodologies (Feigner *et al*, *Proc. Nat'l Acad. Sci USA* 84, 7413 (1987); Schreier, *Liposome Res.* 2, 145 (1992)). Commercial preparations, such as Lipofectin (Life Technologies, Inc., Gaithersburg, Md. USA), are readily available. The amount
5 of liposomes to be administered are optimized based on a dose response curve. Feigner *et al.*, *supra*.

Other suitable liposomes that are used in the methods of the invention include multilamellar vesicles (MLV), oligolamellar vesicles (OLV), unilamellar vesicles
10 (UV), small unilamellar vesicles (SUV), medium-sized unilamellar vesicles (MUV), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV), multivesicular vesicles (MVV), single or oligolamellar vesicles made by reverse-phase evaporation method (REV), multilamellar vesicles made by the reverse-phase evaporation method (MLV-REV), stable plurilamellar vesicles (SPLV),
15 frozen and thawed MLV (FATMLV), vesicles prepared by extrusion methods (VET), vesicles prepared by French press (FPV), vesicles prepared by fusion (FUV), dehydration-rehydration vesicles (DRV), and bubblesomes (BSV). The skilled artisan will recognize that the techniques for preparing these liposomes are well known in the art. (See COLLOIDAL DRUG DELIVERY SYSTEMS, vol.
20 66, J. Kreuter, ed., Marcel Dekker, Inc. 1994).

Preferably, the compound is administered in drops, vapour, atomised droplets, or by nanoparticles. Other forms of delivery particle, for example, microspheres and the like, are also contemplated for delivery of the compound.
25 Formulation of the compound or a liposome or other vesicle comprising said compound in suitable aqueous solvent for delivery by eye-dropper to the eye is particularly preferred.

Guidance in preparing suitable formulations and pharmaceutically effective
30 vehicles, are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, chapters 83-92, pages 1519-1714 (Mack Publishing Company 1990) (Remington's), which is hereby incorporated by reference.

The modulators identified using the methods described herein are useful for the therapeutic or prophylactic treatment of diseases associated with aberrant pericyte function, such as, for example, aberrant cell growth or contractility, retinopathy and other disorders of the eye associated with impaired pericyte function.

Accordingly, a related embodiment of the invention provides a method for treating a subject having impaired pericyte function comprising administering to the subject an amount of a pharmaceutical composition comprising a compound that modulates pericyte function and a pharmaceutically acceptable carrier, diluent or excipient, wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function.

Preferably, the compound is administered under conditions sufficient to alleviate one or more impaired pericyte functions, including impaired pericyte contraction. The alleviation may be temporary, in which case repeated or continuous dosage of the compound may be required. Those skilled in the art will readily be in a position to determine an effective dosage regimen for the active ingredients identified in the inventive screens described herein.

For therapeutic applications (or *in vivo* diagnostic applications for that matter), it is preferred that the compound is administered to the subject's eye, more preferably the retina, in an amount sufficient to modulate pericyte contractile state of the pericyte. Such an amount can be determined empirically, preferably using animal models. The effective amount of the compound will vary according to factors such as the type of disease of the individual, the age, sex, and weight of, and the extent of retinal disease of the individual. Administered concentrations can be adjusted to provide the optimum diagnostic response. For example, several divided doses can be administered separately. Alternatively, combinations of compounds can have synergistic effects and the concentrations can be adjusted accordingly.

Preferably the non-steroidal anti-inflammatory drug is administered in a concentration of about 10 micromol/L to about 300 micromol/L. Preferably, the non-steroidal anti-inflammatory drug N-phenylanthranilic acid is administered in a concentration of about 10 micromol/L to about 300 micromole/L. Preferably, 5 the non-steroidal anti-inflammatory drug flurbiprofen is administered in a concentration of 0.1 micromol/L to about 3 micromole/L. Preferably, the vasoactive peptide PACAP is administered in a concentration of 0.1 micromol/L to about 1 micromole/L. Preferably, the vasoactive peptide VIP is administered in a concentration of about 0.1 micromole/L to about 1 micromole/L.

10

The compounds identified in the novel screens of the invention, especially those agonists of pericyte function, are particularly useful in the diagnosis of impaired pericyte function in a subject. Compounds that have been shown by *In vitro* screening to modulate pericyte function can be tested for safety and 15 efficacy in animal models, such as for example, dogs or rats or pigs, and then proceed to clinical trials in humans, if desired. Naturally, for veterinary applications, no clinical trial in humans is required. Those compounds that are safe and efficacious in animals or humans can be applied to the eye of an appropriate subject to test for normal or healthy retina function. For subjects 20 suffering from impaired pericyte function, such as, for example, glaucoma, corneal angiogenesis, retinopathy of prematurity, or diabetic retinopathy, agonist compounds will generally either fail to elicit a contraction when applied to the surface of the eye, or alternatively, elicit a slowed or incomplete contraction compared to the contractile state achieved for a healthy subject 25 under similar or identical conditions.

Accordingly, a third aspect of the invention provides a method of diagnosing impaired pericyte function in a subject comprising administering to the subject an amount of a compound that modulates pericyte function under conditions 30 sufficient to modify the contractile state of a pericyte and determining the change in the contractile state of the subject's pericytes, wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is indicative that the compound modulates pericyte function. 35 Preferably, the change in the contractile state of the subject's pericytes is

determined by a comparison with the change in contractile state of a pericyte of a healthy subject.

In a related embodiment, the present invention provides a method of
5 diagnosing retinal capillary damage in a subject, the method comprising:

- (i) administering to the subject an amount of a compound that modulates the contractile state of a pericyte wherein said compound is identified by a process comprising determining a change in the contractile state of a pericyte in the presence of said compound, wherein said change is
10 indicative that the compound modulates pericyte function; and
- (ii) detecting dilation or constriction of a capillary in the retina of the subject, wherein a slow or unsubstantial dilation or constriction of the capillary indicates retinal capillary damage.

- 15 Preferably, the change in the contractile state of the subject's capillaries is determined by a comparison with the change in contractile state of a retinal capillary of a healthy subject.

Preferably, the method comprises contacting the subject's eye with an effective
20 amount of the compound suitable formulated for veterinary or pharmaceutical use.

In one embodiment the compound is selected from the group consisting of:
pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive
25 intestinal polypeptide (VIP), a compound having activity on phospholipase C (PLC), a compound having activity on protein kinase A (PKA), a compound having activity on ion-channel hyperpolarisation channels, or a non-steroidal anti-inflammatory drug (NSAID). Preferably, the non-steroidal anti-inflammatory drug is N-phenylanthranilic acid or flufenamic acid or flurbiprofen. More
30 preferably, flurbiprofen will be the R-isomer form.

Preferably the non-steroidal anti-inflammatory drug is administered in a concentration of about 10 micromol/L to about 300 micromol/L. Preferably, the non-steroidal anti-inflammatory drug N-phenylanthranilic acid is administered in
35 a concentration of about 10 micromol/L to about 300 micromole/L. Preferably, the non-steroidal anti-inflammatory drug flurbiprofen is administered in a

concentration of 0.1 micromol/L to about 3 micromole/L. Preferably, the vasoactive peptide PACAP is administered in a concentration of 0.1 micromol/L to about 1 micromole/L. Preferably, the vasoactive peptide VIP is administered in a concentration of about 0.1 micromole/L to about 1 micromole/L.

5

Preferably, a modulation is detected using an ophthalmoscope or Fundus camera.

10 The subject may have one or more symptoms associated with diabetes mellitus, such as, for example, glaucoma, corneal angiogenesis, or diabetic retinopathy. Alternatively, the subject may have no visible symptoms developed in respect of diabetes mellitus or is otherwise considered healthy. Preferred healthy subjects will have normal blood glucose concentrations for their age, ethnicity, sex, and weight, preferably about 5 millimole/L.

15

It is preferred to administer to the subject being tested (healthy or non-healthy) an amount of the compound that is sufficient to induce a dilation of the retinal capillary blood vessels of a healthy subject. Such capillary dilation is a consequence of relaxation of the pericytes, which relaxation enhances the cross-sectional diameter of the retinal capillary vessels. Preferably, the increase in capillary diameter also leads to an alteration in the velocity of blood flow in the capillaries.

25 In the case of a subject having elevated blood glucose levels compared to a healthy subject, or alternatively, a subject suffering from early stage or advanced diabetes mellitus, the compound of the invention induces a reduced dilation of the retinal capillary blood vessels compared to the dilation observed for a healthy subject. Thus, the degree of pericyte relaxation induced for a diseased subject, or a subject having a propensity to develop glaucoma, corneal angiogenesis, or retinopathy is much reduced compared to a normal subject.

35 The present invention clearly contemplated a comparison between the subject being tested and a standardised or normalised response for a healthy subject, thereby obviating the need for a direct side-by-side comparison to reach a diagnosis. To determine a standardised or normalised response for a healthy

subject, the time and extent of dilation of the retinal capillary are determined for a particular compound administered to a panel of healthy subjects having no familial history or symptoms of diabetes mellitus, glaucoma, corneal angiogenesis, retinopathy of prematurity, diabetic retinopathy or ocular disease
5 and the mean responses of the panel are determined. Such epidemiological data are particularly useful for comparison to the response of a test subject to a particular compound being used in the diagnostic assay.

The foregoing embodiments apply *mutatis mutandis* to assays conducted *in*
10 *vivo* using compounds that induce constriction of the retinal capillaries.

Preferably, the dilation response of the retinal capillary blood vessels in response to the compound has a threshold of sensitivity for subjects having blood glucose concentrations up to about 7.5 millimole/L, which includes at the
15 higher end of the scale those subject having diabetic retinopathy. Accordingly, the present assay is particularly useful for diagnosing subjects in the early stages of developing diabetic retinopathy.

The present invention is further described with reference to the following non-
20 limiting examples

Example 1. Preparation of silicone rubber substrate

A small volume of dimethylpolysiloxane (Sigma Chemical Co.) of either
25 60,000cps (DMPS-60M) or 12,500cps (DMPS-12M) viscosity was applied to 12mm diameter glass cover slips. In some cases an intermediate viscosity dimethylpolysiloxane (30,000cps) was prepared by blending 54% by weight of 60,000cps dimethylpolysiloxane with 46% by weight of 12,500cps dimethylpolysiloxane. The coated cover-slip was heated for 2 seconds using a
30 Bunsen burner to induce cross-linking of the surface of the dimethylpolysiloxane and formation of a thin silicone rubber sheet bonded to the cover slip. After preparation, the cover slips were placed in 24-well tissue culture dishes and sterilised by UV irradiation overnight.

Example 2. Growth of pericytes on silicone rubber substrate

Pericytes from primary cultures (first passage) were plated on the cover slips in DMEM supplemented with 10% FCS. The experiments were performed 48h later, when almost all the cells were spontaneously in a contracted state as manifest by wrinkles in the rubber sheet beneath the cells (figure 1).

All experiments were performed using only first passage cultures of pericytes to minimise any change in pericyte physiology that might occur in prolonged culture.

Example 3 Evaluating the contractility of pericytes

The response of the cells to the antagonists was evaluated using phase contrast microscopy at room temperature. The spontaneous contractile tone of pericytes induced tension wrinkles that could be observed after 24h (figure 1). Wrinkles were only measured when associated with a pericyte, as shown in figure 1. Cells were identified as relaxed when the tension wrinkles associated with them diminished in size, and completely relaxed when the wrinkles disappeared. Conversely, a pericyte contracted when there was an increase in the number and length of the wrinkles associated with the pericyte. During each experiment, an image of the pericytes was captured (videocamera and framegrabber) by the computer every minute. The wrinkles were analysed after the experiment from these captured images.

In each of the experiments, the length of each clearly discerned wrinkle associated with the cell was measured 3 times, and the average of the lengths was tabulated. The two ends of a wrinkle were determined by the contrast difference from the background on the picture. The contrast settings on the computer, videocamera or framegrabber were set at the commencement of an experiment and then not changed during the course of an experiment. Similarly, the focus of the microscope was set at the commencement of the experiment and then not changed during the course of an experiment. The depth (level) of solution in the bath was also kept constant, by aspirating and injecting each new solution at the same rate. Thus the only change in the image of the wrinkle (contrast, length) was due to the tension induced in the

silicone substrate by the pericyte. The wrinkles during each experimental condition were counted from the photographs with help of a Zeiss videoplan.

The contractile state of the pericyte was quantified by counting the number of wrinkles (N) and the length of each wrinkle (l) in the thin shin sheet of silicone. From these observations an index of contractility (C_i) was developed from $N \times l$. An experiment to determine the effect of vasoactive agents was conducted by first quantifying C_i with the pericyte in physiological buffer ("control"). The C_i was quantified after exposing the pericytes to a vasoactive agent and allowing the pericyte to reach a steady-state level of contractility, which usually took 10 minutes. The effect of the vasoactive agent was determined by dividing by the C_i in the control condition and multiplying by 100 to obtain an index of effect (I_e , %) that was either less than 100 (pericyte relaxed relative to control) or greater than 100 (pericyte contracted relative to control). As a further control, incubations with buffer alone for the complete duration of exposure to each vasoactive agent were performed in conjunction with each experiment.

Example 4 Testing the functionality of the single-cell contractility assay

Vasoactive agents might modulate the contractile activity of the cells adhering on the silicone substrate. It was confirmed that retinal capillary pericytes are contractile cells, and that the silicone substrate system could provide quantification of the contractility, by observing that norepinephrine, one of the most potent biogenic vasoconstrictors, caused a contractile response.

25

For this purpose cells were grown on silicone rubber. On the day of experiment the cells were rinsed with HEPES-buffered solution for 20 min at room temperature. Then the pericytes were exposed to norepinephrine (N5785, Sigma, USA) at different concentrations ($10^{-6}M$, $10^{-5}M$, $10^{-4}M$) by a rapid exchange of the entire solution. The pericytes were exposed to each new drug concentration for a total of 10 minutes, with an image of the pericytes taken every 1 min. Changes in the number of wrinkles associated with the pericytes were analysed from the images recorded every 1 min.

35 After calculating the index of effect, the experiment demonstrated that the contractility assay was capable of measuring changes in pericyte contractility

and that pericytes were capable of contracting in a dose-dependent manner to norepinephrine (figure 3).

Example 5: Effect of vasoactive peptides on pericyte contractility

5

The inventors have identified several potential vasoactive agents that can affect retinal capillaries. The vasoactive peptides subject to the present experiments are VIP (vasoactive intestinal polypeptide) and PACAP (pituitary adenylate cyclase activating polypeptide). The inventors have identified that these
10 peptides can influence the contractile state of retinal pericytes, an effect that will influence capillary haemodynamics.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic peptide, having a potent action in stimulating cyclic adenosine 3'-
15 monophosphate (cAMP) production in anterior pituitary cells. PACAP and VIP receptors are widely distributed, occurring in the central nervous system and peripheral organs, such as the eye.

The present inventors explored how PACAP and VIP affect capillary tone and
20 thus play a role in vascular regulation through effect of pericyte contraction and relaxation. Experiments determined that pericytes are capable of responding to PACAP and VIP stimulation and *in vivo* can modulate microvessel lumina diameter and thus regulate local blood flow.

25 For this purpose cells were grown on silicone substrate. On the day of the experiment the cells were rinsed with HEPES-buffed physiological solution, and left in this solution for 20 min at room temperature. PACAP or VIP was added in increasing concentrations by fluid exchange over pericytes grown on silicone substrates. Pericytes were exposed for 10 minutes to each new concentration
30 of PACAP or VIP. The effect of PACAP on pericytes was investigated and compared to that of VIP. The concentrations of PACAP were 10^{-9} M, 10^{-8} M, and 10^{-7} M. The concentrations of VIP were 10^{-9} M, 10^{-8} M, and 10^{-7} M.

The time course of the effect of a single dose of PACAP was studied when
35 PACAP 10^{-8} M was applied for 20 minutes. After 20 minutes the solution

containing the drug was removed, and the cells were washed with drug-free buffer. VIP and PACAP were purchased from AUSPEP (Australia).

5 The inventors identified that in one embodiment an increase in cAMP following the binding of PACAP or VIP could result in an increase in cAMP-dependent protein kinase activity. Cyclic 3',5'-adenosine monophosphate (cAMP) is an important intracellular second messenger in many tissues and mediates the effect of multiple drugs and hormones. cAMP regulates a number of different cellular processes such as cell growth and differentiation, ion channel
10 conductivity, synaptic release of neurotransmitters, and gene transcription. Reversible protein phosphorylation is a key regulatory mechanism in eukaryotic cells.

The present inventors further identified protein kinase A (PKA) as being
15 affected by the increase in cAMP following the binding of PACAP or VIP. For this purpose cells were grown on silicone substrate and contacted by a 10^{-8} M concentration of PACAP. The drug adenosine 3',5'-cyclic monophosphothioate, Rp-isomer (Rp-cAMPS, A7850 Sigma USA) is a specific inhibitor of PKA activity and was added sequentially into the PACAP 10^{-8} M solution in three
20 separate concentrations (10 μ M, 30 μ M, and 100 μ M) commencing with the lowest concentration. The pericytes were allowed to reach a steady-state in the altered contractile response (10 minutes) with each Rp-cAMPS concentration before changing the superfusing solution for the next concentration. We discovered that Rp-cAMPS, in a dose-dependent manner and with an EC_{50}
25 value of 26 μ M, inhibited the relaxing effect of 10^{-8} M PACAP. It was shown that the drug N-(2-[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide (H-89, B1427 Sigma USA) at a concentration of 0.3 μ M, which is another specific inhibitor of PKA activity, inhibited the relaxing effect of 10^{-8} M PACAP.
See Figure 6.

30

The present inventors have also shown that the relaxing effect of PACAP on pericyte contraction was in part mediated by the intracellular pathway that mobilises Ca^{2+} from intracellular stores. The drug 1-[6-([(17 β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,2-dione (U73122, U6756 Sigma
35 USA) at a concentration of 10 μ M, is a specific antagonist of intracellular

pathways linked to phospholipase C (PLC), and is an antagonist of the PACAP-induced relaxation of pericytes.

5 Example 6: Effect of non-steroidal anti-inflammatory drugs on pericyte contractility

The inventors have identified several potential non-steroidal anti-inflammatory drugs (NSAID) that can affect retinal capillaries. The NSAID subject to the
10 present experiments are N-phenylanthranilic acid, flufenamic acid and flurbiprofen. The R-isomer form of flurbiprofen was used in these experiments, and is referred to as R-flurbiprofen. The inventors have identified that these NSAID can influence the contractile state of retinal pericytes, an effect that will influence capillary haemodynamics.

15

The present inventors explored how N-phenylanthranilic acid, flufenamic acid and R-flurbiprofen affect capillary tone and thus play a role in vascular regulation through effect of pericyte contraction and relaxation. Experiments determined that pericytes are capable of responding to N-phenylanthranilic
20 acid, flufenamic acid and R-flurbiprofen stimulation and *in vivo* can modulate microvessel lumina diameter and thus regulate local blood flow.

For this purpose cells were grown on silicone substrate. On the day of the experiment the cells were rinsed with HEPES-buffed physiological solution, and
25 left in this solution for 20 min at room temperature. N-phenylanthranilic acid, flufenamic acid or R-flurbiprofen was added in increasing concentrations by fluid exchange over pericytes grown on silicone substrates. Pericytes were exposed for 10 minutes to each new concentration of N-phenylanthranilic acid, flufenamic acid or R-flurbiprofen. The concentrations of N-phenylanthranilic
30 acid were 100 micromole/L, 300 micromole/L and 1000 micromole/L. The concentrations of flufenamic acid were 30 micromole/L, 50 micromole/L, 100 micromole/L and 300 micromole/L. The concentrations of R-flurbiprofen were 0.1 micromole/L, 0.3 micromole/L, 1 micromole/L and 3 micromole/L.

See figures 8, 9, and 10

35

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered
5 in all respects as illustrative and not restrictive.

Dated this 7th day of June 2002

University of Technology, Sydney
By F B RICE & CO
Patent Attorneys for the Applicant:

Phase-contrast micrograph of pericytes contracting a silicone rubber substrate

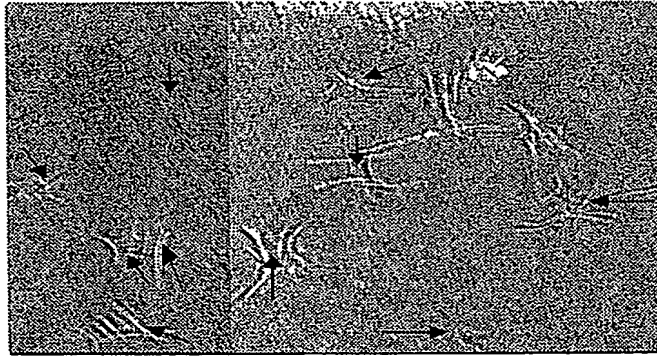
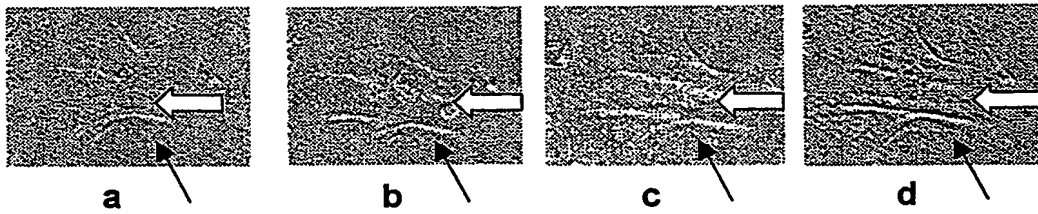
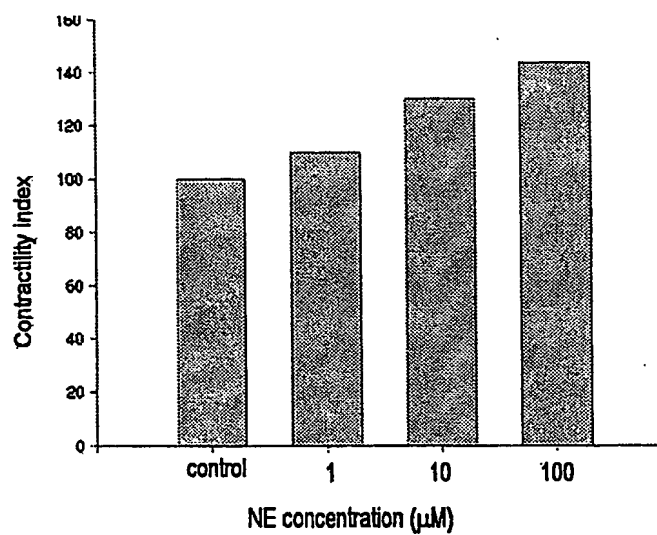


Figure 1

5



10 **Figure 2**



Dose-dependent effect of norepinephrine on contraction of retinal pericytes. Norepinephrine intensified the number of silicone wrinkles in a contraction-dependent manner, inducing development of the contractile tone of the pericytes. Norepinephrine at concentrations greater 10^{-6} M caused contraction with maximal contractions recorded at 10^{-4} M norepinephrine. Norepinephrine induced pericyte contraction of silicone substrate in a dose-dependent manner ($EC_{50} = 8 \mu M$).

Figure 3

Figure 4

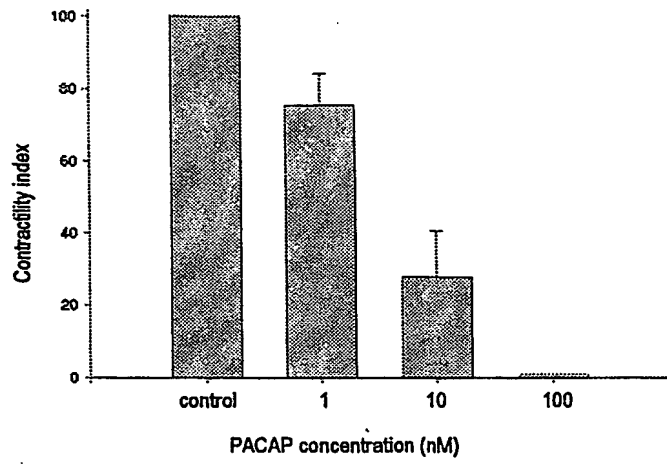


Figure 5

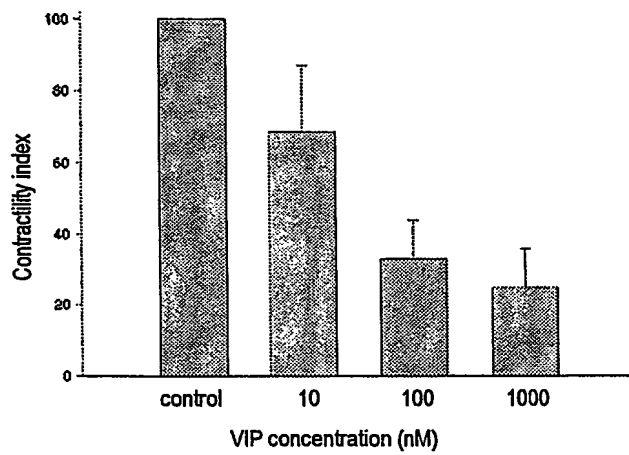


Figure 6

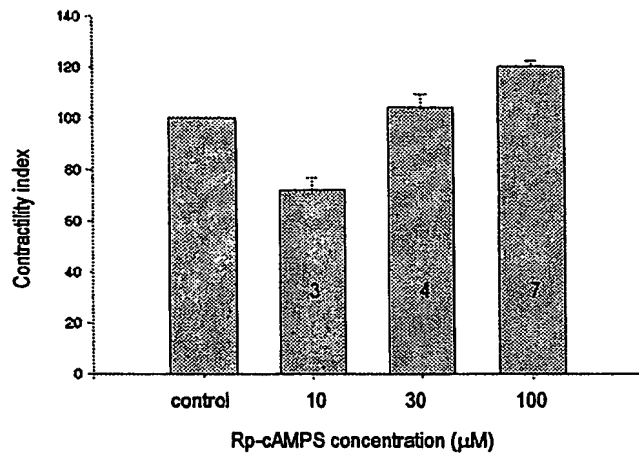


Figure 7

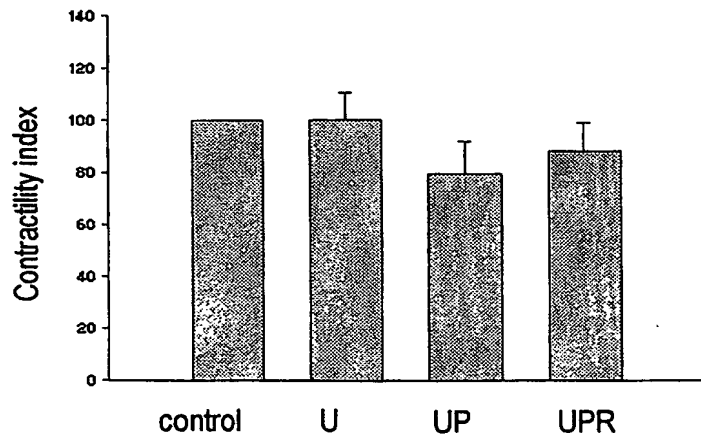


Figure 8

5/7

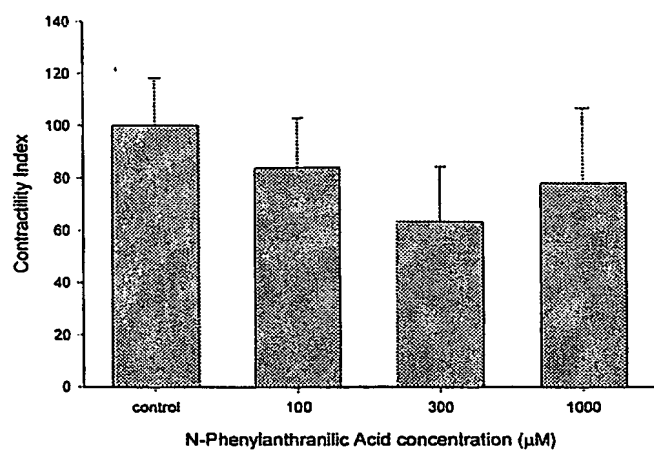


Figure 9

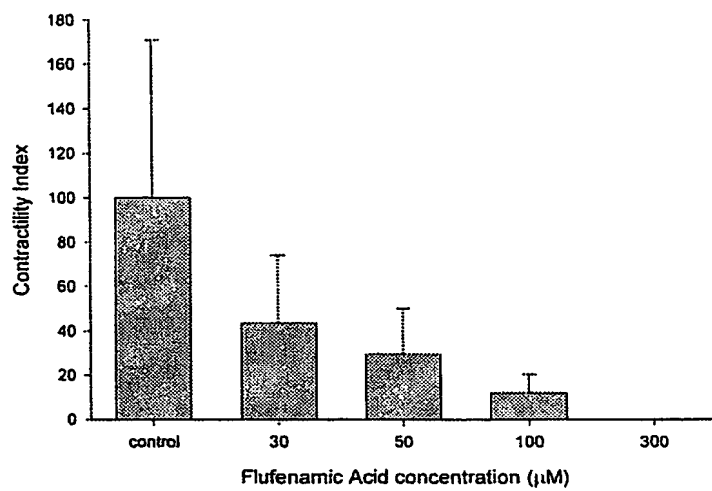
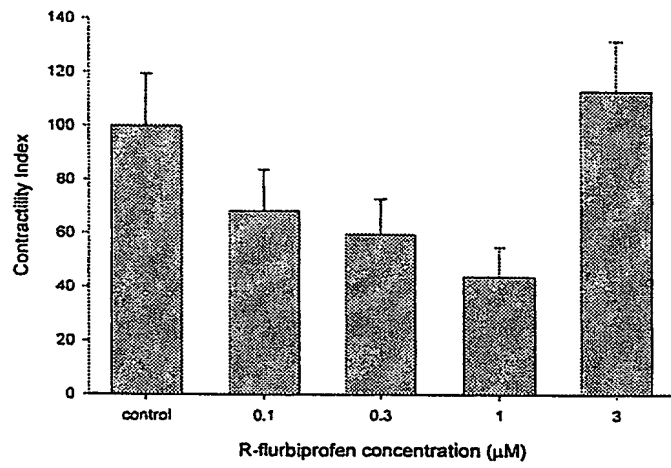


Figure 10



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.